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Benzimidazole F167Y polymorphism in the canine hookworm, *Ancylostoma caninum*: Widespread geographic, seasonal, age, and breed distribution in United States and Canada dogs

Christian M. Leutenegger [a,*], Michelle D. Evason [a], Jennifer L. Willcox [a], Haresh Rochani [a], Holly L. Richmond [a], Cathy Meeks [a], Cecilia E. Lozoya [a], Jeffrey Tereski [a], Samantha Loo [a], Kelly Mitchell [a], Jan Andrews [a], Christian Savard [b]

[a] *Antech Diagnostics, Fountain Valley, CA, USA*
[b] *BioVet Inc., Saint-Hyacinthe, Québec, Canada*

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ABSTRACT

Surveillance data for *Ancylostoma* spp. and the *A. caninum* benzimidazole treatment resistance associated F167Y polymorphism using molecular diagnostics was obtained in a large population of dogs from the United States and Canada. Real-time PCR (qPCR) for *Ancylostoma* spp. and allele-specific qPCR detecting a single nucleotide polymorphism (SNP) F167Y was used in 262,872 canine stool samples collected between March and December of 2022. *Ancylostoma* spp. was found at an overall prevalence of 2.5% (6538/262,872), with the highest prevalence in the Southern US, 4.4% (4490/103,095), and the lowest prevalence in Canada 0.6% (101/15,829). The *A. caninum* F167Y polymorphism was found with the highest prevalence (13.4%, n = 46/343) in the Western US and the lowest in Canada at 4.1% (4/97). The F167Y polymorphism was detected every month over the 10-month collection period. Seasonal distribution showed a peak in June for both *Ancylostoma* spp. (3.08%, 547/17,775) and *A. caninum* F167Y (12.25%, 67/547). However, the *A. caninum* F167Y polymorphism prevalence was highest in September (13.9%, 119/856). Age analysis indicates a higher prevalence of both hookworm infections and occurrence of resistant isolates in puppies. The breeds with the highest F167Y polymorphism prevalence in *Ancylostoma* spp. detected samples were poodles (28.9%), followed by Bernese Mountain dogs (25%), Cocker spaniels (23.1%), and greyhounds (22.4%). Our data set describes widespread geographic distribution of the *A. caninum* benzimidazole resistance associated F167Y polymorphism in the United States and Canada, with no clear seasonality compared to the *Ancylostoma* spp. prevalence patterns. The F167 polymorphism was present in all geographic areas with detected hookworms, including Canada. Our study highlights that the F167Y polymorphism is represented in many dog breeds, including greyhounds.

1. Introduction

The risk for widespread anthelmintic drug resistance in companion animals, in contrast to livestock and horses, has only recently been described with the detection of resistance to all three major drug classes used to treat parasitic nematodes of dogs in the United States (US) (Jimenez Castro et al., 2019). In most of these cases, the F167Y (TTC > TAC) single nucleotide polymorphism in the *Ancylostoma caninum* β-tubulin isotype 1 gene polymorphism was associated with treatment resistance to benzimidazole anthelmintics (Kitchen et al., 2019; Jimenez Castro et al., 2019; Venkatesan et al., 2023). Subsequently, more cases of benzimidazole treatment resistant hookworms in greyhounds were shown to harbor multiple anthelmintic drug resistance (MADR) *A. caninum*, Jimenez Castro et al., 2021; Jimenez Castro et al., 2022). Anecdotal awareness of treatment-resistant hookworm outside the US arose years before the initial reports of MADR resistant hookworms in 2019. Pyrantel treatment failure of *A. caninum* was reported in Australia for the first time in 1987 (Jackson et al., 1987) with several other subsequent reports (Hopkins et al., 19888, Hopkins and Gyr, 1991; Kopp et al., 2007) and benzimidazole resistance in Brazil (Furtado et al., 2014; Furtado and Rabelo, 2015). In the US, hookworm larvae recovered from an infection originally isolated from a retired racing greyhound from

* Corresponding authorAntech Diagnostics, Molecular Diagnostics R&D 17620 Mt. Herrmann Street Fountain Valley, CA 92708, USA.
E-mail address: christian.leutenegger@antechmail.com (C.M. Leutenegger).

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Florida in 2017, was used in a controlled efficacy study where high levels of resistance were confirmed to all drug classes; fenbendazole, pyrantel pamoate and milbemycin oxime yielded efficacies of 26%, 23% and 9%, respectively (Jimenez Castro et al., 2022). These resistant hookworm cases were paralleled by a steady increase in canine hookworm frequencies reported between 2012 and 2018, with an overall increase of 45.6% over the seven-year period (Drake and Carey, 2019). More recent reports also confirm that many resistant *A. caninum* isolates are described in non-greyhound dog breeds (Jimenez Castro et al., 2022 Venkatesan et al., 2023; Leutenegger et al., 2023a). Taken together, these observations indicate a wider than expected spread of resistant hookworm isolates and further highlight the need for readily available, and affordable, drug resistance screening tests for veterinary wellness programs (Jimenez Castro et al., 2022; Marsh and Lakritz, 2023).

Historically, anthelmintic drug resistance testing was based on *in vitro* (e.g., larval development assay, larval motility test), and in vivo fecal egg count reduction tests. These types of tests are time and resource intensive and are impractical in the veterinary clinic for for mass wellness screening. Currently, molecular diagnostics offers multiple protocols to detect the genetic mutations of benzimidazole resistance on the β-tubulin isotype 1 gene. These molecular protocols include conventional Sanger sequencing, pyro sequencing (Von Samson-Himmelstjerna et al., 2009), deep sequencing using next generation sequencing (NGS), RFLP-PCR (Furtado et al., 2018) and allele-specific PCR (Schwenkenbecher et al., 2007). The latter protocol has been used successfully in monitoring all three SNPs conferring benzimidazole resistance in the β-tubulin gene at codons 167, 198 and 200. While no elevated levels of the resistance associated alleles were reported in the canine hookworms in 2009, only a small number of adult hookworms (n = 10) from Georgia, Athens, USA and one pool of adult hookworms obtained from the North Carolina State University, Raleigh, USA were tested (Schwenkenbecher and Kaplan, 2009).

With the confirmed presence of the anthelmintic drug resistance genetic marker F167Y polymorphism in the greyhound dog population and recent reports of anthelmintic resistant isolates in the general dog population (Jimenez Castro et al., 2022; Venkatesan et al., 2023; Leutenegger et al., 2023a), the design of control strategies is needed. These efforts are indicated to maintain the effectiveness of current drugs and monitor for the emergence of resistance to additional anthelmintic compounds, and will require diagnostic protocols for mass screening in pet wellness settings. We recently reported the validation of a hydrolysis probe adapted allele-specific qPCR test with high analytical specificity for the F167Y SNP (TTC > TAC) (Leutenegger et al., 2023a). When using qPCR tests for allelic discrimination, the analytical specificity is of greatest importance as cross-reactivity with the wildtype allele would falsely indicate presence of benzimidazole resistance. Cross-reactivity with the wildtype allele was shown to be highly unlikely: the discrimination power was greater than 10 Cp values (crossing points), indicating that an excess of more than 1770-fold wildtype over mutant allele would have to be present in a sample to cause a false positive result (Leutenegger et al., 2023a). Spike-in experiments confirmed that this scenario is unlikely to occur; samples with the highest detectable parasite burden detected during the clinical validation study, which included over 500 *Ancylostoma* spp. detected samples, did not yield any false-positive results with the F167Y mutation specific hydrolysis probe. Those high burden hookworm samples were confirmed by Sanger sequencing and only contained the wildtype allele TTC. Positive TTC > TAC mutation signals were only detected once synthetic DNA containing the TAC mutation was spiked into those samples. These experiments confirmed the high analytical specificity and that the benzimidazole resistance marker F167Y detected in this surveillance study represented true positive results. The spike-in experiments also confirmed that the optimized total nucleic acid extraction protocol from fecal sample preparations produced inhibition-free material. An aspect of great importance as fecal samples are recognized to be a sample type matrix containing numerous types of PCR inhibitors (Shieh et al., 1995).

qPCR is known to have high analytical sensitivity and is used in many molecular applications as a reference method. The analytical sensitivity of the 167Y mutation specific qPCR was 4 molecules per reaction, lower than the commonly required analytical sensitivity of 10 molecules per reaction. The *Ancylostoma* spp. specific qPCR also had a high analytical sensitivity, which translated into a limit of detection of 6.33 eggs per gram feces (Leutenegger et al., 2023a).

The objective of this fecal surveillance study was to analyze a subset of canine data collected from diagnostic sample submissions to a reference laboratory, and to determine the prevalence of *Ancylostoma* spp. and the *A. caninum* F167Y benzimidazole polymorphism. Additional objectives were to describe the geographic distribution, seasonality, age, and breed associations of *Ancylostoma* spp. and the *A. caninum* 167Y polymorphism using a validated and published molecular diagnostic qPCR test (Leutenegger et al., 2023a).

2. Materials and methods

2.1. Data collection

A subset of detected canine results for *Ancylostoma* spp. (n = 6541) and *A. caninum* F167Y (n = 715) were collected between March and December 2022, from the laboratory information management system of a reference laboratory (Antech Diagnostics, Inc.) as part of a larger parasite molecular diagnostic PCR panel (KeyScreen® GI Parasite PCR, Leutenegger et al., 2023b). Canine ages were divided into: puppies aged less than a year, young adults aged 1–3 years, mature adults aged 4–7 years, and senior dogs aged over 7 years. Geographically, the United States data was divided as per the US Census Bureau into 4 regions: the West, Midwest, South and Northeast, as is commonly described (https://data.census.gov/). This geographical division has been previously used in other prevalence survey studies (Little et al., 2009; Venkatesan et al., 2023).

2.2. Real-time PCR tests and quality controls

The data set for this study was obtained with a commercially available GI parasite molecular test, which identifies 20 individual parasites, the F167Y polymorphism of *A. caninum,* and *Giardia duodenalis* with zoonotic potential (KeyScreen® GI Parasite PCR, Antech Diagnostics, Inc., (Leutenegger et al., 2023b). In addition, two quality controls were used in conjunction with every diagnostic sample. First, an internal positive control (IPC) was used containing a synthetic and random DNA sequence construct spiked into the guanidinium isothiocyanate lysis solution at known quantities. A specific qPCR allowed to detect the synthetic construct after total nucleic acid extraction and a comparison to the known quantity spiked-in initially allowed an assessment of presence or absence of PCR inhibition. A second quality control quantified the amount of amplifiable nucleic acid at both the RNA and gDNA level using a housekeeping gene pan-bacterial 16 S qPCR adapted from previously published protocols (Windsor et al., 2006).

2.3. Statistical analysis

The statistical software package SAS 9.4 was employed to perform various data analysis tasks, such as descriptive statistics to summarize the main features of the variables to analyze the data. We computed the prevalence of each category and the regional distribution of the observations. The prevalence of *Ancylostoma* spp. and *A. caninum* 167Y were estimated, and 95% confidence intervals were computed where appropriate. The results of the descriptive statistics are presented in the following section.

3. Results

3.1. Regional distribution of Ancylostoma spp. and A. caninum F167Y

A total of 262,872 canine fecal submissions were evaluated over the 10-month period in 2022. Out of the total, 6541 (2.5%) samples were found to be positive for *Ancylostoma* spp. Of those 6329 samples contained adequate geographic and demographic information and were used for further analysis and mapping (Fig. 1 A. Prevalence of *Ancylostoma* spp. was highest in the South with 4442 detected cases (4.5%) and lowest in the West with 283 detected cases (0.6%), Table 1) %. The state of Florida had the highest prevalence of *Ancylostoma* spp. positive cases at 26.82% (1698/6329), followed by Texas with 21.91% (1387/6329). In Canada, substantial numbers of submissions were obtained from Alberta (n = 2384) and Ontario (n = 12,717), with *Ancylostoma* spp. prevalence of 0.5% in Alberta (13/2384) and 0.7% in Ontario (83/12,717).

Out of 6329 *Ancylostoma* spp. positive samples (Figs. 1A), 715 (11.3%) were found to have the *A. caninum* F167Y benzimidazole polymorphism (Fig. 1B). In the US, the prevalence of the *A. caninum* F167Y benzimidazole polymorphism was highest in the west with 14.8% (42/283) followed by the south with 11.6% (517/4442) (Table 1). In Florida, there were 30.1% (215/715) *A. caninum* positive cases recorded, followed by 16.92% (121/715) cases in the state of Texas. In Canada, a single *A. caninum* was detected in Alberta with the F167Y polymorphism (7.7%, 1/13), while three others were detected in Ontario (3.6%, 3/83) leading to an overall F167Y polymorphism prevalence in Canada of 4.1% (Fig. 1B).

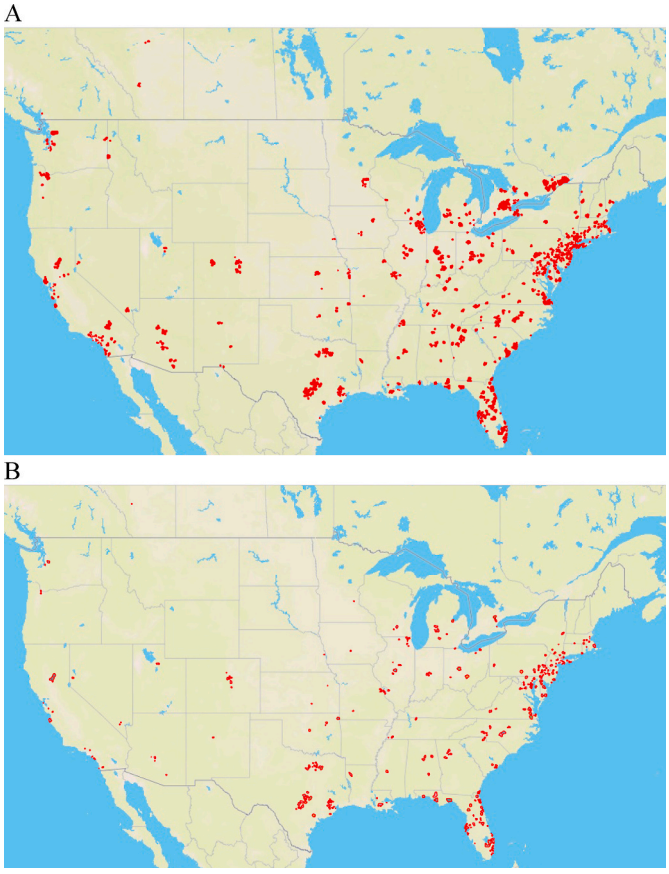

Fig. 1. A: Distribution of *Ancylostoma* spp. in the US. B: Distribution of benzimidazole resistance associated F167Y polymorphism in *A. caninum* in the US and Canada.

Table 1
Regional distribution of *Ancylostoma* spp. and *A. caninum* F167Y polymorphism in the US by census regions.

US Region	*Ancylostoma* spp.		*A. caninum* F167Y	
	n =	%	n =	%
Northeast	1022	1.8%	97	9.5%
Midwest	582	1.8%	59	10.1%
South	4442	4.5%	517	11.6%
West	283	0.6%	42	14.8%
Total	6329	2.5 %	715	11.3 %

3.2. Seasonal prevalence distribution by month

Month by month total numbers and prevalence of *Ancylostoma* spp. and *A. caninum* F167Y in the US are shown in Figs. 2 and 3 A & B. The majority of *Ancylostoma* spp. cases were reported in the last quarter of the year, with December, October, and November accounting for the highest number of cases at 1,116, 992, and 912, respectively. Peak prevalence of *Ancylostoma* spp. occurred during the summer months of June (3.1%), July (2.9%), and August (2.7%), while the lowest prevalence was observed during the winter months of November (2.1%) and December (2.2%) (Fig. 3A). The months of September, November, and December showed the highest prevalence of A. *caninum* with the F167Y polymorphism. The prevalence of the F167Y polymorphism during these months was recorded at 13.9%, 13.5%, and 12.9% respectively (Fig. 3B).

3.3. Age distribution

The highest prevalence of *Ancylostoma* spp. was found in puppies at 3.8% (95% CI: 3.7%–3.9%), followed by young adult dogs at 2.5% (95% CI: 2.4%–2.7%, Table 2). The most common age group submitted for intestinal parasite testing was puppies (<1 y), accounting for 34.3% of the submissions (Table 2 and Fig. 4). *Ancylostoma* spp. infections in puppies (<1 y) accounted for 52.7% of the total positive cases. Young adults (1-4 y) had the second highest frequencies of *Ancylostoma* spp., with 1538 (23.7%) of the positive cases. Mature adults (4-7 y) and senior (=>7) had the lowest prevalence of infections, with 11.8% of the positive cases, each. Senior dogs had the lowest prevalence of *Ancylostoma* spp. at 1.1% (95% CI: 1.0%–1.2%). Prevalence of *A. caninum* F167Y

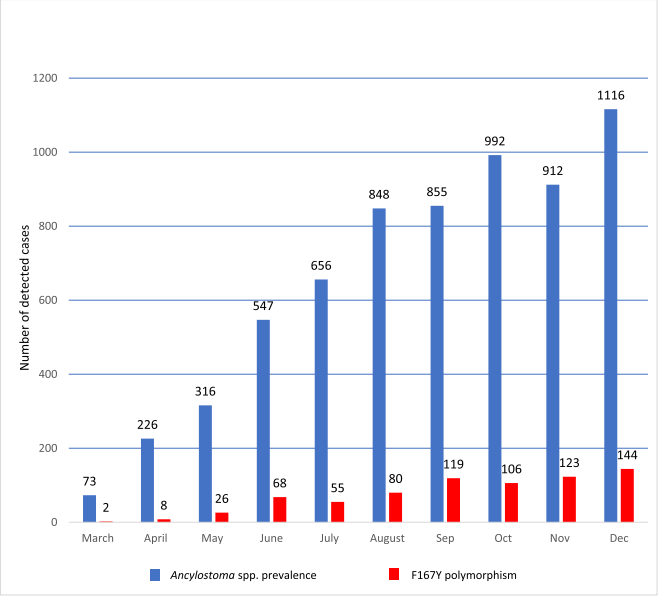

Fig. 2. Total numbers of *Ancylostoma* spp. and *A. caninum* with the F167Y polymorphism by month from March through December 2022.

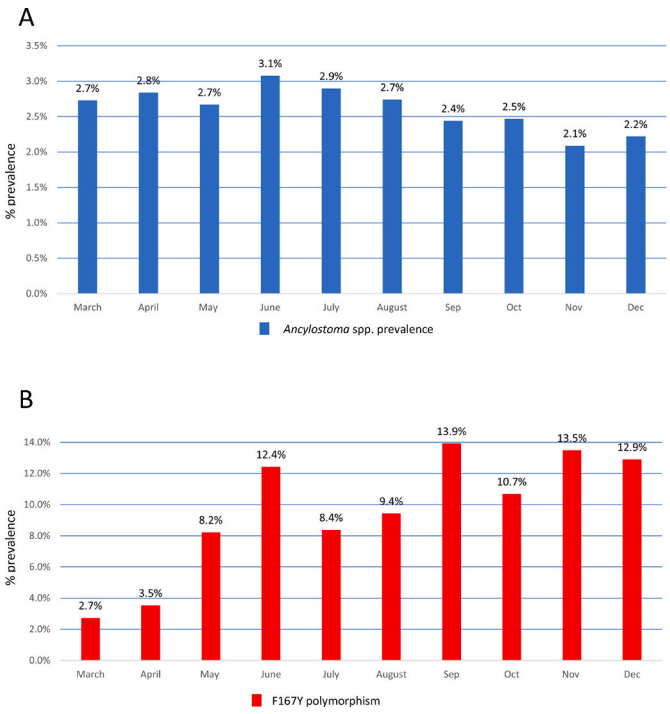

Fig. 3. A: Prevalence of *Ancylostoma* spp. by month. B: Benzimidazole resistance F167Y polymorphism in *A. caninum* by month from March through December 2022.

polymorphism were highest in puppies (<1 y) compared to young adult dogs (1-3 y) with 497/3420 (14.5%) and 135/1538 cases (8.8%), respectively (Table 2 and Fig. 4). *A. caninum* F167Y polymorphism prevalence gradually decreased as dogs reached maturity, with the lowest prevalence observed in the senior age class with 41 cases (5.2%).

3.4. Breed distribution of Ancylostoma spp. and A. caninum F167Y polymorphism

Table 3 displays the prevalence of *Ancylostoma* spp. among the top 20 dog breeds, based on the fraction of submitting cases testing positive. Mixed breed dogs accounted for most of the total submissions and total cases (22.6%). Table 4 shows the top 20 of 72 dog breeds for the F167Y polymorphism, with the top 4 breeds being Poodles (28.9%), Bernese Mountain dogs (25%), cocker spaniels (23.1%), and greyhounds (22.4%). A graphic comparison between *Ancylostoma* spp. and F167Y polymorphism prevalence for 20 dog breeds is shown in Fig. 5. Fig. 6 displays graphically the 20 dog breeds with the highest prevalence of the F167Y polymorphism (data from Table 4) in descending fashion.

4. Discussion

To the authors' knowledge, this study describes the largest population of US and Canada canine fecal samples tested for *Ancylostoma* spp. and *A. caninum* F167Y polymorphism using molecular qPCR tests. A total of 262,872 samples submitted from across the US and Canada, between March and December 2022 were included in the analysis, of which 6541 were detected for *Ancylostoma* spp. (2.5%). In this canine hookworm population, the F167Y polymorphism associated with benzimidazole resistance was detected in 715 samples or 11.3%. Our prevalence findings are considerably higher than those reported in an earlier study, which consisted of 328 individual dog fecal samples and 65 eggs pools from 357 samples with low egg numbers (Venkatesan et al., 2023). However, the samples in that study were based on zinc sulfate centrifugal flotation identified canine hookworm samples and then selected based on purified egg enumeration; therefore, exact prevalence and association of the resistance prevalence with the US hookworm prevalence may not have been possible. Like our work, this

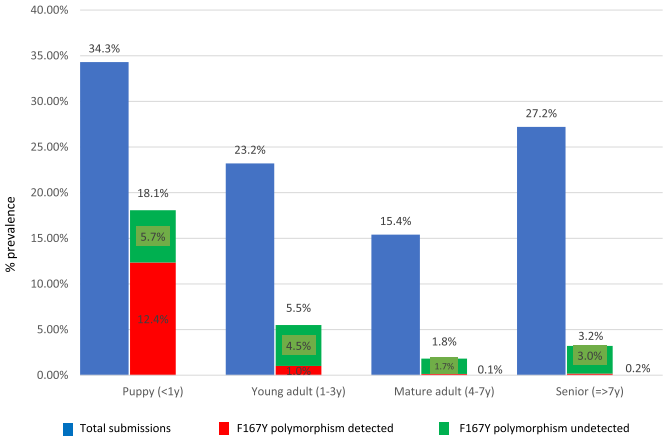

Fig. 4. Age categories with percentage of total number of submissions (blue), the prevalence of detectedF167Y polymorphism detected (red) and the prevalence of F167Y polymorphism undetected (green). The red bar (detected F167Y polymorphism) and green bar (F167Y polymorphism undetected) together represent the prevalence of *Ancylostoma* spp. in each age category.

Table 2
Number of total submissions, *Ancylostoma* spp. and the *A. caninum* F167Y polymorphism in the canine age groups compared to the total sample set or within each age class.

Age Category	Total Submissions		*Ancylostoma* spp.			*A. caninum* F167Y		
	n =	%	n =	%[a]	%[b]	n =	%[a]	%[c]
Puppy (<1y)	89,833	34.3%	3420	52.7%	3.8%	497	68.3%	14.5%
95% CI		34.1–34.4%		51.5–54.0%	3.7–3.9%		64.9–71.7%	13.4–15.8%
Young adult (1-3y)	60,888	23.2%	1538	23.7%	2.5%	135	18.5%	8.8%
95% CI		23.1–23.4%		22.7–24.7%	2.4–2.7%		15.7–21.4%	7.4–10.3%
Mature adult (4-7y)	40,243	15.4%	765	11.8%	1.9%	55	7.6%	7.2%
95% CI		15.2–15.5%		11.0–12.6%	1.8–2.0%		5.6–9.5%	5.5–9.3%
Senior (=>7y)	71,243	27.2%	768	11.8%	1.1%	41	5.6%	5.2%
95% CI		27.0–27.3%		11.1–12.7%	1.0–1.2%		4.0–7.3%	3.8–7.1%

CI= Confidence interval.

[a] Percentage of number of detected dogs within the respective age class as the numerator and the total of dogs reported for *Ancylostoma* spp. or *A. caninum* 167Y polymorphism as denominator.

[b] Percentage of number of detected dogs within the respective age class as the numerator and the number of total submissions for *Ancylostoma* spp. within each age class as denominator.

[c] Percentage of number of detected dogs within the respective age class as the numerator and the number of total positive for *Ancylostoma* spp. within each age class as denominator.

Table 3
Ancylostoma spp. prevalence calculated by dog breed. The prevalence of the top 20 breeds, based on the % positivity of *Ancylostoma* spp. is listed.

Breed	Total submissions by breed		*Ancylostoma* spp. prevalence	
	N	%	N	%
Mixed breed	40,514	20.8%	1043	22.6%
Labrador retriever	16,252	8.4%	401	8.7%
German shepherd	7612	3.9%	336	7.3%
Australian shepherd	5882	3%	168	3.6%
Golden retriever	10,017	5.2%	162	3.5%
Greyhound	746	0.4%	161	3.5%
Poodle	5775	3%	121	2.6%
Beagle	3451	1.8%	115	2.5%
Boxer	3152	1.6%	106	2.3%
Chihuahua	8194	4.2%	94	2%
American Staffordshire	1631	0.8%	89	1.9%
Bulldog	1783	0.9%	85	1.8%
Siberian husky	3185	1.6%	77	1.7%
Great Pyrenees	995	0.5%	76	1.6%
French bulldog	5272	2.7%	73	1.6%
Great Dane	1435	0.7%	71	1.5%
Dachshund	5137	2.6%	68	1.5%
Border collie	2622	1.6%	66	1.4%
Doberman pinscher	1309	0.7%	65	1.4%
Rottweiler	1592	0.8%	63	1.4%

study convincingly showed that resistant canine hookworms have become geographically widespread in the US and are primarily in non-greyhound dog breeds.

Other molecular methodologies to identify canine hookworm benzimidazole resistance mutations include conventional Sanger or deep sequencing protocols (Kitchen et al., 2019; Jimenez Castro et al., 2019). These techniques can reliably detect genetic markers that are associated with drug resistance. However, cost and time to result limit their use to confirmatory or research testing, and these methods are likely unsuitable as everyday screening tests. Other methods of hookworm benzimidazole resistance detection, such as *in vitro* anthelmintic drug susceptibility testing and serial fecal egg count reduction tests may be impractical, inconvenient and/or too costly for screening and surveillance purposes. Of the available test options, fecal qPCR tests are probably best suited for high throughput diagnostic laboratories in both human and veterinary medicine, due to their affordability, reagent robustness, sophisticated equipment, and quick turnaround time, which predestines these for surveillance applications in addition to routine wellness testing in clinical practice (Massetti et al., 2020).

These data also demonstrate detection of the *A. caninum* F167Y polymorphism in geographic regions not previously highlighted in prior studies. New regions with notable concentrations of the canine hookworm resistance marker detection were identified in canine samples from New Hampshire to Virginia extending to the eastern flank of the Appalachian Mountains. This is unusual compared to other human and pet dog population density in these regions, where resistant hookworms are more likely found in concentrated clusters or hotspots compared to a more widespread occurrence. Examples of the focal clusters we observed were the major metropolitan centers in states like Texas, that included Houston, Dallas, and Austin. Another interesting finding in this study were the cases of resistant hookworm that appeared to be spread along I-60 between Oklahoma City and St. Louis with a focal cluster in St. Louis. Other regional and sometimes widespread geographic clusters were found in Florida, with smaller clusters in Southern California, Colorado, Oregon, and Chicago.

The *Ancylostoma* spp. prevalence distribution is consistent with previous reports, indicating lower frequencies in northern geographies, which are likely due to climatic and environmental factors (Little et al., 2009). Regional distribution of the F167Y polymorphism and a higher prevalence in the western US (14.8%) as compared to the South, Midwest, and Northeast in the US was observed in this study and has been reported before (Venkatesan et al., 2023). The finding of higher F167Y polymorphism prevalence contrasts to the relatively low prevalence of overall *Ancylostoma* spp. in the West. Possible explanations are restrictions in the accessible refugia or an overall smaller size of refugia for hookworm in the more arid western environment, which has been discussed as an important factor to accelerate development of anthelmintic resistance (Von Samson-Himmelstjerna et al., 2021).

Another novel observation in this study was the detection of the *A. caninum* 167Y polymorphism in Canada, indicating northward expansion of benzimidazole resistance, specifically into Ontario and Alberta (Fig. 1B). Interestingly, clinical history revealed that three of these four cases originated from the US, including an adopted racing greyhound from Oklahoma, a shepherd mix imported from the Southern US, and a Walker hound presumed to be imported from the US. A case series of dogs in Canada with the F167Y polymorphism, some of which lacked a travel history, indicated that BZ resistance is endemic in the

Table 4
A. caninum F167Y polymorphism total number, prevalence and percentage of *Ancylostoma* spp. detected with the F167Y polymorphism by dog breed.

Breed	Total of submissions by breed	F167Y polymorphism detected	Prevalence of F167Y polymorphism detected by submission	*Ancylostoma* spp. with F167Y polymorphism by breed
	n =	n =	%	% (167Y/n)
Poodle	5775	35	0.6%	28.9% (35/121)
Bernese Mountain dog	1655	7	0.4%	25.0% (7/28)
Cocker spaniel	1789	6	0.3%	23.1% (6/26)
Greyhound	746	36	4.8%	22.4% (36/161)
Bulldog	1783	18	1.0%	21.2% (18/85)
Boston terrier	2001	7	0.4%	18.4% (7/38)
Boxer	2001	7	0.4%	18.4% (7/38)
French bulldog	5272	13	0.3%	17.8% (13/73)
Cane Corso	541	8	1.5%	17.0% (8/47)
Beagle	3451	18	0.5%	15.7% (18/115)
Chihuahua	8194	13	0.2%	13.8% (13/94)
Golden retriever	10,017	22	0.2%	13.6% (22/162)
Maltese	3730	4	0.1%	12.9% (4/31)
Great Dane	1435	9	0.6%	12.7% (9/71)
Dachshund	5137	8	0.2%	11.8% (8/68)
American Staffordshire terrier	1631	10	0.6%	11.2% (10/89)
Doberman pinscher	1309	7	0.5%	10.8% (7/65)
Mixed breed	40,514	106	0.3%	10.2% (106/1039)
Australian shepherd	5882	17	0.3%	10.1% (17/168)
Labrador retriever	16,252	37	0.2%	9.2% (37/402)

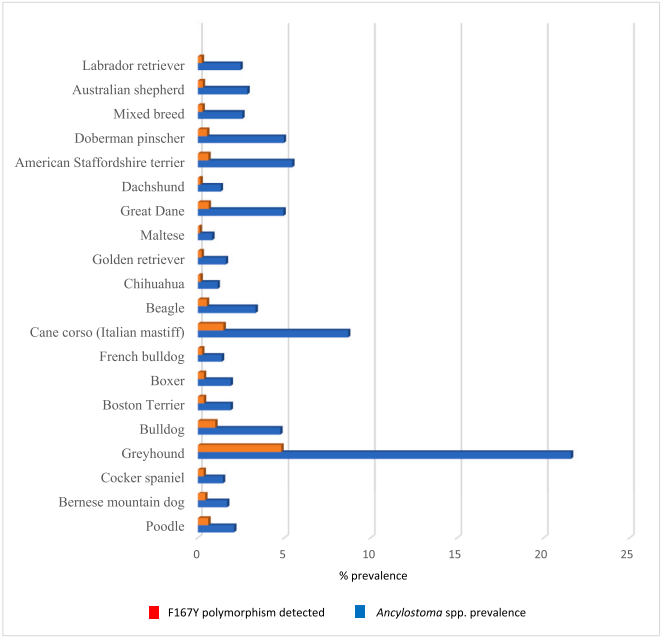

Fig. 5. Breed distribution of *Ancylostoma* spp. (blue bars) and F167Y polymorphism prevalence (red bars) in 20 dog breeds.

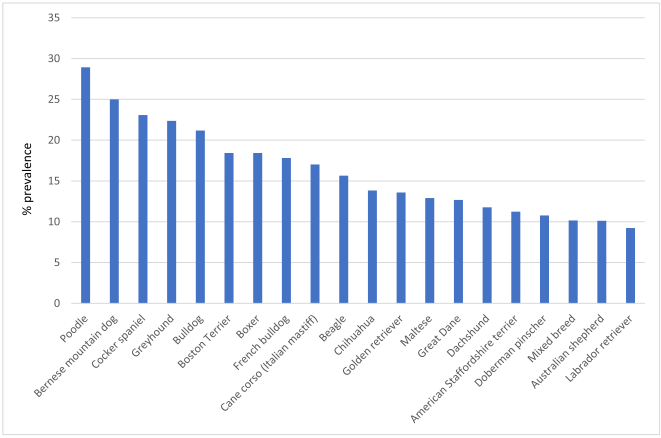

Fig. 6. Comparison of *A. caninum* F167Y polymorphism prevalence within each dog breed shown in Fig. 5.

country (Evason et al., 2023). Prior to our work, a single Canadian case of a suspect treatment resistant Blue Lacy dog imported from Texas had been reported in 2007 (Wojnarowicz and Smith, 2007). Importation, adoption, and rehoming of pet dogs from regions in the US with higher endemicity rates of resistant hookworms into Canada is a plausible explanation for the presence of resistant parasites or vector-borne infectious agents in geographic regions where they are not expected to complete their life cycles or have not been reported in the past (Al Izzi et al., 2013; Nezami et al., 2023).

Seasonality has been previously demonstrated to play a role in parasite prevalence in the United States and Canada and was included in the analysis of this population. *Ancylostoma* spp. prevalence was found to increase in the warmer months and decrease in the cooler months, as has been reported before (Drake and Carey, 2019). Interpretation of the F167Y polymorphism in canine hookworms in the early months of sampling is more difficult due to the small number and possible sampling bias. In May and in following months, frequencies of F167Y were consistently above 8% and as high as 13.9% in September of 2022. Certain isolated weeks during the study period had frequencies as high as 25.1% in southern geographic locations such as Florida. With the continued availability and increased utilization of testing, future studies will be necessary to evaluate whether statistical association can be identified with seasonality and the F167Y polymorphism prevalence.

The commercially available test used in this study represents the first surveillance test for the detection of the *A. caninum* benzimidazole F167Y polymorphism for use in routine fecal samples. Previously reported diagnostic methods used purified egg preparations (Venkatesan et al., 2023), which entails a cumbersome and time-consuming protocol to concentrate egg from a sample. This technique includes several centrifugation, wash, and filter steps, as well as freeze-thaw cycles and incubation steps for >12 h and ultimately precludes its use in a high throughput reference laboratory. Purified egg preparations do provide a highly concentrated starting material and therefore it is likely that, in combination with the deep sequencing protocol, higher frequencies of F167Y mutation can be detected, such as the 49.7% described in Venkatesan et al. (2023). For this reason, a possible limitation of this study is that the prevalence of the F167Y polymorphism in the US and Canadian hookworm populations may have been underestimated with standard fecal samples utilized for nucleic acid extraction. While protocols could likely be developed to increase the sensitivity of F167Y polymorphism detection, it should also be acknowledged that these adjustments may lead to increased detection of low allele frequency samples which do not correlate with a resistance phenotype, as has been shown (Venkatesan et al., 2023). Only a direct comparison between the allele-specific qPCR and the egg enrichment along with deep sequencing protocols would provide clarity of the comparability of diagnostic performance between the two protocols.

Breed analysis confirmed that the F167Y polymorphism can be found throughout the companion pet dog population. In our data, greyhound dogs were neither most diagnosed with *Ancylostoma* spp. nor most likely to contain the F167Y polymorphism in samples positive for *Ancylostoma* spp., indicating that the F167Y polymorphism has spread not only geographically, but also throughout many dog breeds. In this study, the F167Y polymorphism was found in 72 dog breeds including mixed breed dogs. While greyhounds showed a high percentage of detected F167Y polymorphism in *Ancylostoma* spp., three dog breeds including poodles, Bernese Mountain dogs and cocker spaniels had a higher percentage of hookworms containing the F167Y polymorphism. The spread of antimicrobial and antiparasitic resistance has long been considered a One Health issue, and this data demonstrates that the F167Y polymorphism is an issue for many breeds and regions, and does not stay confined to one area, group, or breed.

Infection with hookworms was identified in all four age groups. The distribution of *Ancylostoma* spp. across age categories was remarkably similar to those described more than a decade ago (Little et al., 2009). The highest hookworm prevalence was found in puppies (< 1 year), followed by young adult (1–3 y) dogs, and then mature adult (4-6 y) and senior (=>7 y) dogs. Age-dependent prevalence for the presence of the F167Y polymorphism in canine hookworms has not been reported before. Our work describes the highest prevalence for the F167Y polymorphism in puppies, followed by young adult and mature adult dogs, and lowest in senior dogs.

Puppies (<1 y) were the most frequently submitted age class in this study with 34.3% overall parasite prevelance as compared to young adult dogs (1-3 y) with 23.2%, mature adult (4-6 y) with 15.4%, and seniors (>7 y) with 27.2%. When calculated against the total number of dogs infected with hookworm for which age information was available (n = 6491), puppies were more frequently detected with hookworms than any other age class (3,420, 52.7%). Interestingly, the prevalence of the F167Y polymorphism was highest in hookworms detected in puppies with 68.3% and gradually declined over the age classes with the lowest prevalence observed in senior dogs with 5.2%. The higher prevalence of detected F167Y polymorphism in puppies could be explained by the increased use of anthelmintic treatments recommended for puppies in the first year, driving up the selection for survivors with the resistance

mutation (Von Samson-Himmelstjerna et al., 2021) Likewise, senior dogs are likely to be treated less frequently, reducing the prevalence for the selection of the resistance mutation, and increasing the worm population in refugia. It is well known that treatment frequency and treatment strategies affect the rate with which resistance against benzimidazoles develops (Kaplan, 2004).

From a One Health perspective, the implications of widespread anthelmintic drug resistance cannot be underestimated. Prolonged shedding of treatment resistant hookworms, due to missed detection or treatment resistance, leads to a higher level of environmental contamination with resistant hookworm eggs. Consequently, the infection risk and subsequent zoonotic concern for humans increases. Widespread soil contamination in public spaces with zoonotic parasites has been reported recently (Waindok et al., 2022). Parasite diagnostics in people are based on centrifugal flotation which does not detect resistance unless a fecal egg count reduction protocol is being used. Like the early detection of pyrantel treatment resistant canine hookworms, pyrantel treatment resistant human hookworm infections have been reported (Reynoldson et al., 1997). To our knowledge, molecular identification of anthelmintic resistance, such as for the resistance mutations on the beta-tubulin gene is not currently available in people.

Over- or misuse of a single anthelmintic drug does not explain the existence of MADR. The first anthelmintic resistance to a drug shown to exist in hookworm from pet dogs was against pyrantel (Jackson et al., 1987; Reynoldson et al., 1997; Kopp et al., 2007, 2008). In addition, widespread, indiscriminate, and frequent deworming programs have become the standard in veterinary medicine, with the utilization of over-the-counter dewormers consisting of different drug classes. While these programs seemingly had success, livestock and human mass deworming programs have shown limited benefit in overall reduction of parasite frequencies (Allen and Parker, 2016). With the onset of MADR in canine hookworm, an increasing prevalence of hookworm infections in dogs between 2012 and 2018 has been reported (Drake and Carey, 2019). Similarly, an increasing prevalence of heartworm infections between 2013 and 2016 as shown by the CAPC and AHS incidence maps (Drake and Wiseman, 2018), which has been paralleled by macrocyclic lactone resistance in *Dirofilaria immitis* (Geary et al., 2011; Bowman and Manella, 2012; Eisenstein, 2017; Prichard, 2021). It remains to be seen if drug resistance is restricted to these examples, or if there is a general underestimation of the resistance problem with other parasites such as praziquantel resistance in *Dipylidium caninum* (Chelladurai et al., 2018; Loftus et al., 2022), and increasing metronidazole and albendazole resistance in human isolates of *Giardia duodenalis* (Lalle and Hanevik, 2018; Lemee et al., 2000).

The main limitations of this study are related to those inherent to its retrospective nature and limited associated clinical information. As such, home environment, clinical history, presentation, and clinical signs, as well as travel and medication history were unavailable for this population. Particularly, prior anthelmintic use and/or exposure could not be determined from the electronic database. Future analyses and prospective studies will be needed to further elucidate these characteristics. Another limitation of this study is that while the overall sample size was large, some individual comparison groups had a small sample size and thus were not further subdivided. Furthermore, the recently described Q134H SNP associated with benzimidazole resistance (Venkatesan et al., 2023) and added to this PCR test in summer of 2023 was not included in this data set.

5. Conclusions

Surveillance data of *A. caninum* associated beta tubulin isotype-1 codon 167 mutation (F167Y polymorphism) indicates how widespread the F167Y polymorphism is distributed in the US and Canadian pet dog populations. Monitoring and integrated screening strategies using high-throughput molecular diagnostic tests for specific fecal parasites and for genetic drug resistance markers such as the F167Y polymorphism can determine the true extent of geographic, seasonal, breed, and age distribution for suspected benzimidazole resistance. This work provides a basis for ongoing surveillance efforts, raises awareness of One Health and antimicrobial stewardship, and highlights risk factors that aid clinical decision-making and more efficient use of anthelmintic drugs in companion animals.

Authors' contributions

CML, CEL, JT, SL, JA and MDE were involved in study inception, CML, HR, HLR, and JLW developed the study design, data analyses, interpretation, and statistics; CML directed the study. All authors contributed to the writing and editing of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

All authors are employees (CML, MDE, JLW, CM, CEL, JT, SL, KM, JA, CS) or consultants (HR, HLR) for Antech Diagnostics, Inc., Mars Petcare Science & Diagnostics.

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